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General discussion

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7. General Discussion

Inadequate consumption of dietary fibers in the Western world diet coincides with increased immunological disorders such as inflammations, diabetes, obesity, cardiovascular disorders and cancer [1, 2]. Such immunological disorders are considerably lower among native Africans, whose diets consist of large quantities of dietary fibers [1, 2]. Earlier, the beneficial effects of dietary fibers were assumed to be from their fecal bulking effects, however, now dietary fibers are recognized as immunomodulatory agents [1, 3]. One of the most accepted mechanism of dietary fibers' regulatory effect is through the short chain fatty acids (SCFAs). SCFAs are produced by commensal microbiota after fermentation of dietary fibers [4]. SCFAs promote growth of gut epithelial cells and thus promote intestinal barrier functions [5]. SCFAs can also induce anti-inflammatory effects by promoting growth of regulatory T cell population [4, 6, 7]. However, SCFA independent immunomodulatory effects of dietary fibers also exist [8, 9]. This suggests that other mechanisms must be involved in addition to the commensal microbiota dependent effects.

Recently, a new mechanism for immunomodulatory effects of dietary fibers was suggested through direct stimulation or inhibition of immune receptors [8, 9]. This mechanism supports the notion that during the passage through intestine, these dietary fibers, depending on their structure, size and chemical linkages, can be recognized by the immune system. For example, dietary fiber inulin was described to induce activation of Toll-like receptor 2 (TLR2) depending on its chain length [8]. The TLR2 activation capacity of inulin increased with the increase in chain length which led to barrier protective effects in the intestine [8]. In addition to the human nutrition perspective, dietary fibers are also important for animal nutrition. Animal feed has to nutritionally support the growth of the production animals as well as to reduce the infection load in these animals [10, 11]. As, the use of antibiotics in animal feed is proven to be detrimental for humans and animals alike [12], novel strategies are needed to reduce the use of antibiotics but still keep the animals healthy.

In this thesis, we further explore the hypothesis of direct interaction between dietary fibers and the immune system. Innate immune system is the first to recognize any foreign molecules entering the body, and thus we analyzed the interaction of dietary fibers with innate immune receptors known as pattern recognition receptors (PRRs) [13]. Different PRRs recognize different structural patterns as their epitopes. Thus, herein we analyzed different structural features of dietary fibers required to interact with PRRs which can induce or modify the immune-effects of dietary fibers. We studied cereal grain dietary fibers such as β-glucan, arabinoxylan [14] and pectin [15, 16] as these are cheap sources of dietary fibers which can be used in animal feed and in human nutrition. The mechanism of immunomodulatory effects of these

dietary fibers is important to predict their immune effects *in vivo* and thus to design functional feed and food products. In the following sections we will further discuss the new findings presented in this thesis related to the structural features of dietary fibers and its influence on immunomodulatory effects.

7.1 Endoglucanase digestion of oat β-glucan enhanced its immune-reactivity

In chapter 2 we described that the digestion of dietary fiber by commensal microbiota derived enzymes can increase the direct immunomodulatory effects of a dietary fiber [17]. Oat β-glucan was shown to have limited immune-activation potential *in vitro* [18]. However, *in vivo*, immunomodulatory properties of oat β-glucan are very well recognized [19, 20]. We hypothesized that the reason for this difference might be due to enzymatic digestion of oat β-glucans in the large intestine *in vivo*. In support of our hypothesis, we observed that endoglucanase digestion of oat β-glucan increased the Dectin-1 activation capacity of oat β-glucan. We observed that the reason for increased Dectin-1 activation was due to differences in particle size and linkages in non-digested and digested β-glucan. The endoglucanase digestion of oat β-glucans lead to increased β(1-3) linkage exposure on the oat β-glucan and also lead to reduced particle size of oat β-glucan [17].

This study with oat β-glucan demonstrates that the microbiota dependent immunomodulatory effects *in vivo* are not limited to the SCFA and microbiota induced regulatory effects. Digestion of dietary fibers by microbiota can also contribute to enhance the direct immunomodulatory effects as shown in this case for oat β-glucan [17]. The mechanism presented in chapter 2 provides an important link between the microbiota dependent effects and direct immunomodulatory effects through PRRs.

The observations in chapter 2 also present two important structural modifications which can be used as tools to design functional food/feed products containing immune-stimulatory β-glucans. First is, that the increased β(1-3) linkage exposure can increase immune-stimulation through Dectin-1 receptor. Second is, that the lowered particle size of β-glucans, close to 3 μm size, can enhance Dectin-1 activation. The reduced particle size probably allows more efficient clustering of Dectin-1 receptors for enhanced activation [21]. It might be argued that, the observed effect of endoglucanase digested β-glucan was because of either of the above effects. This further needs to be clarified using controls of larger particle sized β-glucans having only β(1-3) linkages. However, with either of the mechanism, endoglucanase digestion of oat β-glucan provides an interesting tool to understand immune-

stimulation through Dectin-1 receptor.

Oats are commonly used in animal feed and the use of endoglucanase digested oat β-glucan to increase immune stimulation can be an innovative method to reduce the use of antibiotics in the animal feed industry [22]. In chapter 2, the immune activation potential of both non-digested and digested oat β-glucan was studied in human dendritic cells. Endoglucanase digested oat β-glucan mainly increased expression of chemokines like RANTES, MCP-1, IL-8, and cytokine IL-4. IL-4 cytokine is known as a meditator for Th2 immune responses [23], wherein MCP-1 can enhance IL-4 expression in T-cells [24]. RANTES has also been demonstrated to stimulate Th2 response, for example in AIDS patients [25]. Thus, it can be suggested that endoglucanase digestion of oat β-glucan mainly stimulates Th2 immune response chemokines. Th2 immune response cytokines and chemokines are responsible for immunity against extracellular parasites and allergic reactions [26]. Thus, endoglucanase digested oat β-glucan could be used as a novel feed additive to enhance immunity against infections.

7.2 Arabinoxylan is a novel activator of Dectin-1 receptor

In chapter 3 we have described arabinoxylan as a new ligand for activation of Dectin-1 receptor [9]. Dectin-1 receptor, as mentioned above is a receptor for β-glucan. β-glucan is composed of β(1-3) and β(1-6) or β(1-4) linked D-glucose units [27]. Arabinoxylan is composed of xylose residues linked by β(1-4) linkages having O-2 and/or O-3 substituted with arabinose [28]. Thus, the backbone of β-glucan and arabinoxylan is composed glucose and xylose respectively. In cyclic conformations, both sugars differ only at carbon atom number 6 wherein, conformations of β(1-4) linked xylose and β(1-4) linked glucose are very similar [29]. Although β(1-4) linked glucans are not recognized by Dectin-1, presence of β(1-4) linkages along with β(1-3) in cereal grain β-glucans are known to activate Dectin-1 receptor [30]. Thus, probably the structural similarity, between arabinoxylan and β-glucan, is responsible for activation of Dectin-1 receptor by arabinoxylan. The activation of Dectin-1 by arabinoxylan also demonstrates that the C-type lectin like domain in Dectin-1 receptor is more versatile than the assumed specificity for β(1-3) linked glucans [31]. Apart from Dectin-1 receptor, CR3 and Lactosylceramide have also been described as β-glucan receptors [32, 33]. It might be possible that arabinoxylan can interact with these receptors.

We have demonstrated in chapter 3 that co-application of both particulate and soluble β-glucan increased expression of IL-4 and IL-23 in dendritic cells. Although, this observation is against the generally accepted role of soluble β-glucan

to inhibit particulate β-glucan induced activation of Dectin-1 receptor [21], similar has also been reported for IL-6 expression in murine dendritic cells wherein combination of soluble and particulate β-glucan treatment lead to increase in IL-6 production [34]. These results suggest a new Dectin-1 stimulatory role for soluble β-glucan instead of only an inhibitory role.

Our finding, that arabinoxylan activates Dectin-1 and modifies the particulate β-glucan induced Dectin-1 activation suggests that *in vivo*, the co-existence of these dietary fibers can lead to competition for binding with Dectin-1 receptor. Thus, different proportions of these dietary fibers may have different immunomodulatory effects *in vivo*. Both arabinoxylan and β-glucan are plant origin dietary fibers and both are present in wheat cell walls [35] and in other cereal grains like oat and barley [36]. Thus, different varieties of wheat, oat or barley containing different proportions of arabinoxylan and β-glucan might have different immunomodulatory effects depending on the dietary fiber contents [36, 37]. Also, we might be able to modulate the immune response by selecting varieties having optimal combination of both arabinoxylan and β-glucan.

7.3 Particulate β-glucan synergistically activates Dectin-1 and TLR4 wherein, TLR4 stimulates a regulatory pathway.

The reason to study role of TLRs in Dectin-1 activation was to confirm whether TLR4 has a role in Dectin-1 activation. Previous studies have suggested that co-stimulation of TLR4 and Dectin-1 led to exponential increase in cytokine production [38]. Similarly, in Candida albicans infection, both Dectin-1 and TLR4 were shown to be responsible for immune stimulation wherein TLR4 knockout mice had increased susceptibility for infections [39]. However, this synergistic role has not been studied in cells expressing both Dectin-1 and TLR4 which are stimulated only with particulate β-glucan. To achieve this aim, in chapter 4, we developed new reporter cell lines which expressed combination of Dectin-1 receptor transcript variants i.e. Dectin-1A and Dectin-1B with TLR4.

Particulate β-glucan could synergistically activate both Dectin-1A-TLR4 and Dectin-1B-TLR4 cells. However, soluble β-glucan could only enhance activation of Dectin-1A-TLR4 and not Dectin-1B-TLR4. It is proposed that particulate β-glucan can enhance immune response through clustering of Dectin-1 receptors, while soluble β-glucan fails to cluster Dectin-1 receptor and shows weaker activation [21]. The different synergistic effects of particulate and soluble β-glucan on Dectin-1 and TLR4 receptors, might be another reason for the different activation potentials of particulate and soluble β-glucan. These observations demonstrate that the structural

features of dietary fibers can influence the synergism between different PRRs and thus can modulate the immune effects of dietary fibers.

The role of TLR4 in Dectin-1 activation was further analyzed by applying TLR4 blocking antibody to particulate β-glucan stimulated human dendritic cells. We observed that TLR4 blocking in particulate β-glucan stimulated dendritic cells could enhance the cytokine production. This observation suggests that TLR4 activates a NF-κB regulatory pathway [40, 41]. The classical NF-κB regulation involves, proteins known as inhibitor of NF-κB (IkB). When IkB is bound to NF-kB subunits, it sequesters them to the cytoplasm and thus inhibit NF- κB activation. When an inflammatory stimulus is received by a cell, the IkB proteins are phosphorylated by IkB kinase (IKK) which leads to ubiquitination of IkB and degradation, releasing NF-κB to enter the nucleus [41]. However, to our knowledge, TLR4 has not been described to inhibit NF-κB. Thus, probably the NF-κB regulatory pathway is specific for Dectin-1 and TLR4 activation through a yet unexplored pathway. The regulatory role of TLR4 also demonstrates that inhibition of TLR4 while inducing immune stimulation by β-glucan could be a novel strategy to further enhance stimulation by β-glucan in immune cells.

7.4 Novel mechanisms for immunomodulatory effects of β-glucan

In this thesis we have shown three different mechanisms to modify the immunomodulatory effects of β-glucan. In chapter 2 we described that the microbiota digestion of β-glucan can increase the binding efficiency of β-glucan with Dectin-1 receptors, thus increasing the immune-stimulation potential. In chapter 3 we showed that the Immunomodulatory effects of β-glucan can be modified in presence of other dietary fibers such as arabinoxylan. Lastly, in chapter 4, we showed that particulate β-glucan can synergistically activate other PRRs such as TLR4 along with Dectin-1 to induce an immune response. Thus, although the Dectin-1 activation by β-glucan is well known, the final immune effect can be modified depending on enzyme digestion by commensal microbiota, interaction with other dietary fibers, and also by synergistic activation of different PRRs. We have demonstrated that the direct immune effects of dietary fibers can be modulated by varied mechanisms and all these mechanism collectively or individually can modify their beneficial effects *in vivo*.

7.5 Pectin binds and inhibits TLR2 in degree of methyl esterification dependent manner

In chapter 5, we demonstrated that the immunomodulatory effects of dietary pectin are through direct binding and inhibition of TLR2. The lemon pectin used in these experiments is mainly composed of α-galacturonic acid chains wherein different degrees of methyl esterification (DM) impart different physical and chemical properties to pectin [16]. The structural simplicity of this pectin, allowed us to explore the true role of structural features in pectin which modify the immunomodulatory effects. To analyze the effect of DM values on immunomodulatory effects of pectin, we tested pectins having different DM levels with reporter cell lines for TLR2, TLR4, and TLR5. Interestingly, we observed a substantial inhibition of TLR2 and only a weak inhibition for TLR4 and TLR5. Although pectin has been shown to inhibit TLR4 dependent inflammation [42], in our assay we did not observed a high level of inhibition by any of the analyzed pectins. These differences may have been due to different origin and chain length of pectin applied in these experiments [42]. The TLR2 inhibition was also dependent on the DM value of the pectin. It was only the low DM pectin that could inhibit TLR2 at the lowest analyzed concentration. Whereas at high concentrations, all the DM values of pectin were able to inhibit TLR2. These observations confirm the role of structure features such as DM values of pectin for their immunomodulatory effects.

The mechanism of TLR2 inhibition by pectin was analyzed by determining the true binding between TLR2 and pectin. In the ELISA assay, we applied the extracellular domain of TLR2 known as TLR2 ectodomain to the ELISA wells having immobilized pectin at the base. The binding of TLR2 ectodomain to pectin was determined by using the hemagglutinin (HA) tag on the TLR2 ectodomain. This ELISA assay could determine the true binding between TLR2 and low DM pectin while the binding efficiency decreased with increasing DM values. Thus, the inhibition of TLR2 was by blocking of the TLR2 ectodomain by pectin. The ELISA assay developed in chapter 5 is an important technological platform to determine the true immunomodulatory effects of dietary fibers through binding with PRRs.

7.6 Low DM pectin binds to TLR2 through electrostatic interactions leading to inhibition of TLR2-TLR1

As mentioned in the previous section, in chapter 5, we demonstrated that the low DM pectin was most efficient in inhibition of TLR2. TLR2 can form a hetero-dimer with other TLRs depending on the ligand presented to the immune cell [43]. TLR2-TLR1 is activated when stimulated with tri-acylated lipopeptide [44], while TLR2-TLR6

is activated when stimulated with di-acylated lipopeptide [45]. Both these receptor combinations with TLR2 have also been shown to activate different activation pathways [43]. In chapter 5, we observed that pectin could only inhibit the pro-inflammatory TLR2-TLR1 activation but not the tolerogenic TLR2-TLR6 activation.

As low DM is a negatively charged molecule, in chapter 6 we hypothesized that the TLR2-pectin binding is mainly through electrostatic interactions. Also, because we could observe inhibition of TLR2-TLR1 by pectin, we hypothesized that the pectin binding site was near the ligand binding site of TLR2 and the interface for TLR2-TLR1 interaction. To confirm this hypothesis we selected four positively charges amino acids R315, R316, R321 and K347 and replaced them with uncharged amino acid glutamine (Q) near the ligand binding site of TLR2 [44]. R321 and K347 were selected as both these amino acids are shown to be involved in TLR2 and TLR1 interface binding [44]. While, R315 and R316 were chosen because these amino acids were structurally closer to the ligand binding groove [44]. These amino acids were selected by using PyMOL program to visualize the TLR2 structure and to ascertain physical proximity of the selected amino acids to the TLR2 ligand binding site. We designed two TLR2 mutants - TLR2 RRQQ (R321 and K347 mutated to Q) and TLR2 QQQQ (R315, R316, R321, and K347 mutated to Q). When we tested the binding between pectin and TLR2 using TLR2 mutants, we observed that low DM pectin could bind less to the TLR2 QQQQ mutant than to TLR2 RRQQ mutant. Thus, reduced positive charges on TLR2 reduced the binding of low DM pectin. However, these observations didn't hold for high DM pectin. From these observations we can conclude that low DM pectin binds to TLR2 through electrostatic interaction.

Another observation from the mutant studies was that the increase in uncharged amino acids in TLR2 mutants assisted in binding of high DM pectin to TLR2. This enhanced binding might be due to increase in hydrogen binding between high DM pectin and TLR2. These observations further reaffirm the importance of the structure of dietary fibers and the nature of interaction with PRRs. Such studies of binding forces between dietary fiber and PRR will allow us to accurately design the dietary fibers targeting the specific sites on PRRs and to use these dietary fibers for therapeutic purposes.

7.7 Pectin lowers doxorubicin-induced ileitis in mice through inhibition of TLR2 and the anti-inflammatory effect is independent of SCFA levels.

To confirm whether the TLR2 inhibition capacity of pectin could be beneficial in inflammations, we analyzed in chapter 5, the effects of pectin in doxorubicin

induced ileitis model in mice. The reason to specifically use this inflammation model was because the doxorubicin induced inflammation has been shown to be reduced in TLR2 knockout mouse [46]. The two main symptoms of doxorubicin induced ileitis, neutrophil infiltration and apoptosis in the crypts could be reduced by pectin administration. Pectin administration to mice could reduce the inflammatory symptoms and the most prominent observation was that, the level of reduced symptoms by pectin was similar to that of TLR2 blocking antibody. The production of proinflammatory cytokines was reduced by pectin in both peritoneum and plasma, showing that the anti-inflammatory effects of pectin are effective in both intestinal and systemic immune system. Furthermore, the anti-inflammatory effects of pectin were independent of SCFA concentrations as pectin administration did not enhance SCFA production in mice. Thus, we concluded that the anti-inflammatory effect of pectin was due to its inhibitory action on TLR2 and not due to SCFA production. These observations serve as proof of principle for our PRR assay, showing that the interactions of dietary fibers with PRRs *in vitro* can be extrapolated to immunomodulatory effects *in vivo*. Also, we can conclude that the direct immunomodulatory effects of dietary fibers can be used as therapeutic measures against inflammations.

The doxorubicin treatment in mice induces cell death in the intestinal crypts, releasing damage-associated molecular pattern molecules (DAMPs). These DAMPs are then recognized by TLR2 resulting in inflammatory reaction in intestine [46]. TLR2 is able to recognize variety of DAMPs such as high mobility group box-1 (HMGB1) [47], heat shock protein 60, and 70 (HSP60 and HSP70) [48, 49]. These DAMPs have been implicated in inflammatory and autoimmune diseases such as rheumatoid arthritis [50] and multiple sclerosis [51]. DAMPs are also implicated in various ageing related diseases such as atherosclerosis and gouty arthritis [52]. Specifically, for gouty arthritis, monosodium urate crystals are known to be recognized as DAMPs by TLR2 to induce an inflammatory response [53]. As the anti-inflammatory effect of pectin were observed in systemic immune system as well, we can suggest that dietary pectin can be used as a novel therapeutic agent to alleviate ageing related inflammatory disorders such as gouty arthritis and support healthy ageing in elderly. The anti-inflammatory effects of pectin also show that the increased intake of dietary fibers can be beneficial against inflammations, and thus dietary fibers can be instrumental in reducing the increased frequency of immunological disorders in western world.

Future perspectives

In this thesis we have further explored the mechanism for direct immunomodulatory effects of dietary fibers. We have shown that the structural features of dietary fibers are responsible for their interaction with PRRs and thus for their immunomodulatory effects. The studies with β-glucan show that the immune-stimulatory effects of dietary fibers can be enhanced by microbiota derived enzymes, by interaction with other dietary fibers like arabinoxylan and also by differential activation of PRRs. These different mechanisms, detailed in this thesis can be used to modulate the immune-stimulation potential of different dietary fibers. By using dietary fiber pectin as a model, we demonstrated that the anti-inflammatory effects of dietary fibers *in vitro* can be extrapolated *in vivo*. The mechanisms described in this thesis can help to design function food or feed products using dietary fibers to improve immunity, to maintain intestinal immune homeostasis and also to use in therapy against immune disorders.

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